UNIVERSITY OF GONDAR

COLLEGE OF MEDICINE & HEALTH SCIENCES

SCHOOL OF PHARMACY

DEPARTMENT OF PHARMACOLOGY

EVALUATION OF ANTI MALARIAL ACTIVITY AND SAFETY OF THE METHANOL EXTRACT OF *HAGENIA ABYSSINICA* AGAINST PLASMODIUM BERGHEI IN SWISS ALBINO MICE.

By

TAFERE MULAW (B.pharm)

ADVISOR :- TEFERE ABULA (PROF)

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As members of examining Board of the Final MSc Open Defense, we certify that we have read and evaluated the thesis prepared by Tafere Mulaw entitled evaluation of anti malarial activity and safety of the methanol extract of Hagenia abyssinica against plasmodium berghei in swiss albino mice and recommended that it be accepted as fulfilling the thesis requirement for the degree of Master of Science in Pharmacology.

1.---------- ---------- ----------

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Final approval and acceptance of the thesis is contingent upon the submission of the final copy of the thesis to the University of Gondar through the Department Pharmacology of the candidate's major department. I hereby certify that I have read this thesis prepared under my direction and recommend that it be accepted as fulfilling the thesis requirement.

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Name of Advisor Signature Date

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Lists of abbreviations

ACTs	Artemisinin based combination therapies
ANOVA	Analysis of variance
CQ	Chloroquine
EHNRI	Ethiopia Health and Nutritional Research Institute
FOMH	Federal Ministry of Health-Ethiopia
UOG	University of Gondar
IC50	Median inhibitory concentration
IP	Intra peritoneal
IV	Intravenous
LD50	Medium lethal dose
OECD	Organization for Economic Co-operation and Development
SPSS	Statistical Package for the Social Sciences
WHO	World Health Organization

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ABSTRACT

Background :- Malaria constitutes one of the major health problems in Ethiopia. One of the reasons attributed for the upsurge is the development of resistance of Plasmodium falciparum and the emergence of multi drug resistant strains of the parasite to anti malarial drugs. A continued search for other effective, safe and cheap plant-based ant malarial agents thus becomes imperative in the face of these difficulties.

Objective :- The objective of the present study is to identify phytochemical profiles , evaluation of acute toxicity, sub acute toxicity and the in vivo anti malarial activities of the methanolic extracts of *Hagenia abyssinica* (Bruce) J. F. Gmel.

Methods :- The plant material *Hagenia abyssinica* was screened phytochemically for presence of secondary metabolites. Acute toxicity studies of the extracts were also carried out by giving up to 2000mg/kg to non infected mice. Weight loss, change in general behavior and mortality were used as indicators of toxicity and also evaluated for its antimalarial activity *in vivo* assays against *Plasmodium berghei* infected mice using 4 day suppression test. Different doses of 100, 200 and 400mg/kg crude extracts, chloroquine base 10mg/kg (positive control) and normal saline (negative control) administered by an intragastric gavage daily for the treatment period.

Results :- Qualitative Phytochemical screening showed presence of Anthraquinones, terpinoids, saponins, phenols, flavonoides, alkaloids and tannins.The crude extract was non toxic at a single dose of 2000mg/kg body weight in acute toxicity study. Antimalarial suppressive test of methanol crude extracts at 100, 200 and 400mg/kg showed 65.29%, 79.6%, 83.33 respectively indicating that the plant is promising for further investigation.

Conclusion:- Crude extracts of *H.abyssinica* caused strong activities against *P. berghei in swiss albino mice* indicating that they contain some chemical constituents that possibly lead to antimalarial drug development. No signs of acute toxicity were observed. Further detailed pharmacological and toxicological studies are recommended for drug development.

1. Introduction

1.1 Background

1.1.1 Pathophysiology of Malaria

Malaria is a febrile hemolytic disease caused by species of protozoa belonging to the genus Plasmodium . There are well over 200 different species of plasmodia and the parasite is capable of infecting many animal species such as reptiles, birds, and various mammals.There are four main *Plasmodium* species that cause disease in humans: *P. falciparum*, *P. vivax, P. ovale* and *P. malariae.* In addition, a simian parasite, *P. knowlesi*, occasionally infects humans (1).
Malaria due to *P. falciparum* is the most deadly form and it predominates in Africa; *P. vivax* is less dangerous but more widespread, and the other three species are found much less frequently. Malaria parasites are transmitted to humans by the bite of infected female mosquitoes of more than 30 anopheline species(2).

1.1.2. Epidemiology of Malaria

Globally, an estimated 3.4 billion people living in 104 countries were at risk of malaria ,and an estimated 207 million clinical cases which lead to nearly 627 000 deaths in 2013 .of those approximately 80% of cases and 90% deaths are estimated to occur in African Region, with children under five years of age and pregnant women most severely affected. malaria accounted for post-neonatal child deaths of 7% globally and 15% in Africa in 2012. Malaria killed more than 1,200 children under 5 years of age every day in 2012. Childhood malaria mortality is strongly concentrated in the poorest countries in sub- Saharan Africa (3).

Malaria economic effect is tremendous and includes direct cost of treatment and prevention, as well as indirect costs such as lost productivity from morbidity and mortality, and time spent seeking treatment . In Africa it is estimated that economic losses due to malaria amount to about $12 billion a year, which by far exceeds the resources needed for malaria control, estimated at about $3 billon .it also reduces growth of GDP per capita by 1.3% per year (4). Malaria is ranked as the leading communicable disease in Ethiopia, accounting for about 30% of the overall Disability Adjusted Life Years lost. According to the FMOH's health and health related indicators report for 2010/11 shows deaths due to malaria in under five years constitute 12.6% compared to 21.1% in 2003/04. WHO estimated that there were 5,400 malaria deaths in Ethiopia

in 2010 among Ethiopian children under five years of age, but recent surveillance reports the annual malaria deaths in 2011 is 550(3,5). Malaria is present everywhere except in the central highlands . FMOH estimates that there are about 12 million suspected malaria cases each year. The FMOH reported a total of 3,384,589 malaria cases from July 2011-June 2012, with (53.0%) of these laboratory confirmed, with (59.2%) *P. falciparum* and (40.8%) *P.vivax*. Ethiopia reported 936 malaria deaths in 2011 (3,6). Over the past years, the disease has been consistently reported as the leading cause of outpatient visits, hospitalization and death in health facilities across the country (7). About 75% of the geographic area of the country has significant malaria transmission risk (defined as areas <2,000 m) which contains 57.3 million (68%) of the 84.3 million population of Ethiopia. The transmission of malaria in Ethiopia depends on altitude and rainfall with a lag time varying from a few weeks before the beginning of the rainy season to more than a month after the end of the rainy season . Malaria transmission peaks bi-annually from September to December and April to May (8).

1.1.3 Life Cycle of Plasmodium

Knowledge of the life cycle of the malaria parasite is fundamental to understanding the methods of prevention, treatment and research pursuits. Although malaria parasites differ from each other in many respects, they have similar basic life cycle. *Plasmodium* species exhibit a heteroxenous life cycle involving vertebrate hosts and arthropod vectors (female Anopheles mosquito). *Plasmodium* species are quite host–specific and there is no zoonosis . The parasite has a complex life cycle and in order to eradicate the disease, Liver stage, Blood stage ,transmission stage, Mosquito stage should be considered for treatment .The life cycle of *Plasmodium* species starts when a malaria parasite infected female Anopheles mosquito bites a healthy individual and inoculates sporozoites (infective form) into the human host. These sporozoites infect liver cells (hepatocytes) within 30 to 40 minutes and mature into schizonts, which rapture the liver cells and are released 10,000 to 30,000 merozoites approximately from 6 to 15 days according to species. Merozoites released from liver cells start to invade red blood cells within 15 to 20 seconds in the blood. However, in *P.vivax* and *P.ovale,* some of the sporozoites remain in the liver and become dormant hyponozoites. This form can remain for months and years until reactivated to complete its life cycle, responsible for secondary erythrocytic schizogony and relapses in patients. In erythrocytes the ring stage trophozoites mature into schizonts which rapture to release

merozoites, after 48- 72 hours depending on the species, which invade and destroy new erythrocytes. The asexual erythrocytic cycle usually continues until controlled by the immune response or chemotherapy or until the patient dies (in case *of P. falciparum*). some merozoites differentiate into sexual erythrocytic stages (gametocytes).(9)

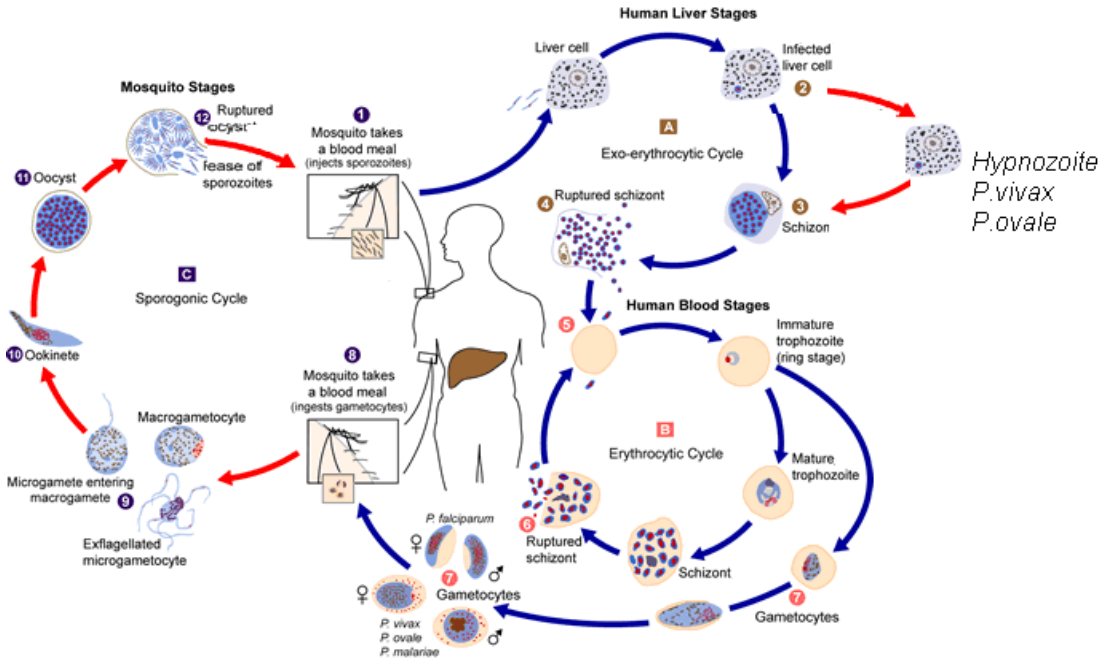


Fig.2 Malaria life cycle (Modified from CDC,2003)

The merozoites enter into the blood circulation,where they infect red blood cells. They further undergo asexual multiplication, some merozoites mature again into schizonts that lead to more merozoites; others differentiate into gamet ocytes which, when picked up by a second mosquito during a further bite, undergo a series of transformations in this second mosquito, leading eventually to the production of new sporozoites that can infect another human being(10).

Within the mosquito midgut, the male gametes will fuse with female gametes to create zygotes . These zygotes develop into motile ookinetes that ultimately become embedded in basal lamina beneath the midgut epithelial wall as oocysts .after several days, a single oocyst develop to a syncytial cell (sporoblast) with thousands of nuclei.In a massive cytokinesis event, thousands of haploid daughter sporozoites assemble from the surface of the mother cell , and these infective sporozoites then migrate to the mosquito salivary glands for transmission to the host (11).

1.1.4 Malaria Prevention and Control

There are a number of approaches towards the prevention and control of malaria and the choice of intervention in a country or region are usually most dependent on cost-effectiveness.

Some of the factors that contributed to high cost control programs, the emergence of new insecticide resistant strains of the vector, creation of new mosquito breeding sites, the problem of drug resistance (*Plasmodium falciparum*) to almost all currently available antimalarial drugs, lack of organized health infrastructures and the migration behavior of people that are increase the incidence and spread of malaria (12).

Prevention and control of malaria is being approached on two fronts: treatment with antimalarial drugs and Vector control. Early diagnosis and treatment with antimalarial drugs is essential in controlling the spread of the disease. By reducing the number of infected humans, the number of infected mosquitoes is effectively reduced. This type of control is especially important when outbreaks of malaria occur (13).

Vector control is achieved by creating a barrier between the human host and mosquitoes, interrupting their life cycle and reducing vector density. Protect people from being bitten by infected mosquito though the use of insecticide-treated nets, insect repellents and indoor residual spraying of insecticides to kill the adult or larval mosquitoes, using natural biological controls such as the introduction of fish which eat the mosquito's larvae or bacteria (*Bacillus thuringiesis*) which excrete larval toxins and reducing mosquito breeding sites (14).

1.1.5 Malaria Treatment and Drug Resistance

Treatment of sick individuals using correct dose of antimalarial drugs interrupted the life cycle of the parasite. Early treatment of cases also reduces transmission by reducing the opportunities for mosquitoes to become infected. Antimalarial drugs that eliminate developing or dormant liver forms are called tissue schizonticides; those that act on erythrocytic parasites are blood schizonticides; and those that kill sexual stages and prevent transmission to mosquitoes are gametocides (15). Few available agents are causal prophylactic drugs (capable of preventing erythrocytic infection). However, all effective chemoprophylactic agents kill erythrocytic parasites before they increase sufficiently in number to cause clinical disease (4). Currently, most approved malaria drugs target only the blood stages of the disease. The two exceptions are the

combinati on of atovaquone /proguanil which is also effective in clearing parasites from the liver, and primaquine. The latter clears not only liver schizonts but also hypnozoites ,the dormant liver-stage parasites in P. vivax and P. ovale infections,thus providing what is known as a radical cure.Antimalarial drugs fall into groups.The first are quinoline based antimalarials,which includes quinine and its derivatives chloroquine (CQ), amodiaquine, premaquine and mefloquine and The second class is the Antifolate compounds that inhibit the synthesis of parasite pyrimidines and thus of parasitic Deoxyribonuclic acid (DNA) . The third class of antimalarial is based on the natural endoperoxide Artemisinin.Artemisinin and its semi synthetic derivatives (dehydroartemisinin (DHA), artemether, arteether, artesunate, artelinic acid are the most rapidly acting and effective against multi-drug resistant strains of the parasite(16).

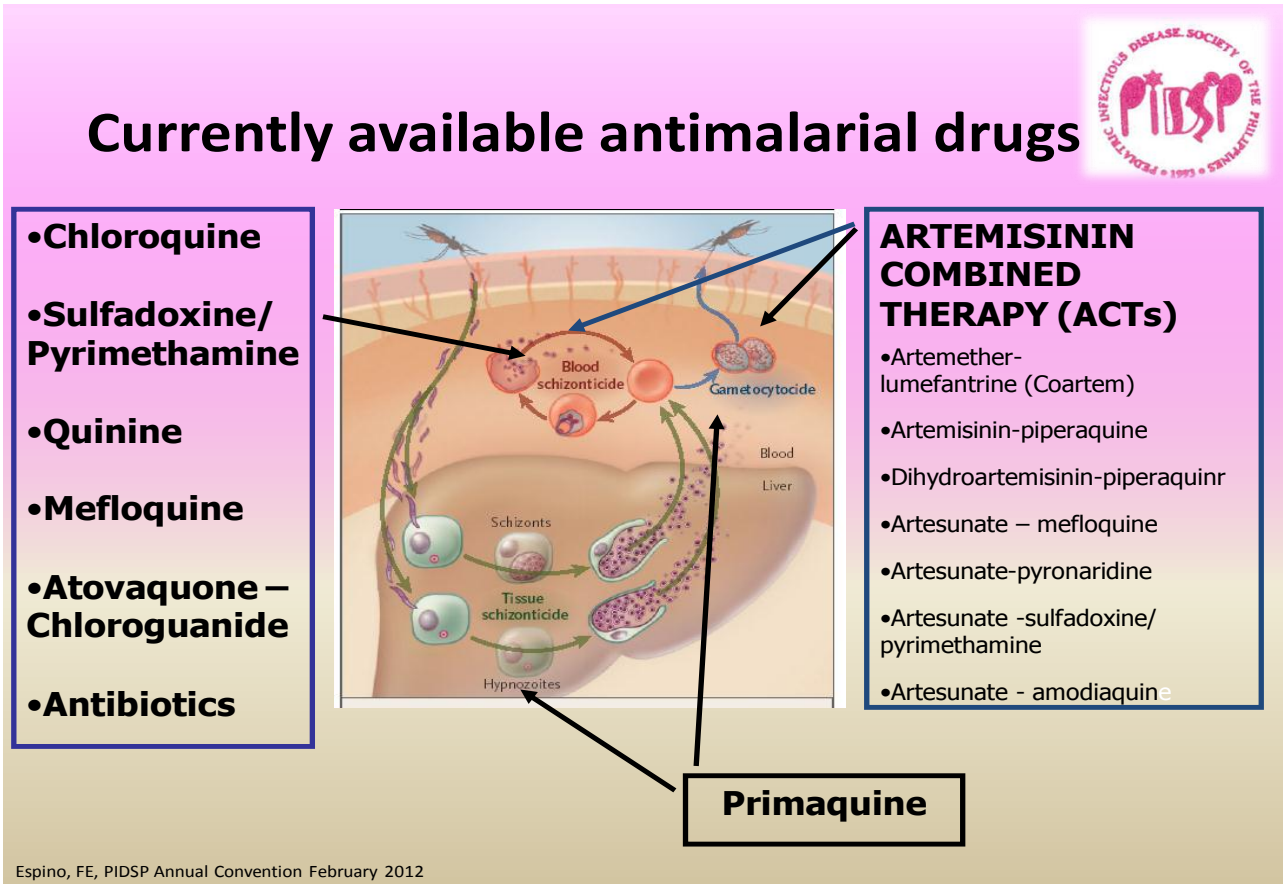


figure. 2 anti malaria drugs site of action

Antimalarial drug resistance is a major concern for the global effort to control malaria(17). Resistance to antimalarial medicines has been documented in all classes of antimalarials, including the artemisinin derivatives, and it is a major threat to malaria control. Widespread and

indiscriminate use of antimalarials exerts a strong selective pressure on malaria parasites to develop high levels of resistance. Resistance can be prevented, or its onset slowed considerably, by combining antimalarials with different mechan *P. falciparum* resistance to artemisinins has been detected in four countries in South East Asia: in Cambodia, Myanmar,Thailand and Viet Nam isms of action and ensuring very high cure rates through full adherence to correct dose regimens(6,8,12,). With the increase in cases of drugs resistance and absence of vaccine, there is an urgent need for new antimalarial drugs with novel modes of action. One approach to this is the investigation of medicinal plants and natural products.

1.1.6 Antimalarial Drugs from Traditional Medicinal Plants

The isolation of bioactive compounds from medicinal plants, based on traditional use or ethnomedical data, is a highly promising potential approach for identifying new and effective antimalarial drug candidates (18). Historically the majority of the drugs have been derived from medicinal plants or structures modeled on plant lead compounds, more than 50% of all drugs in clinical use today contain certain substances of natural origin, most of these products are from plants (19). Previous findings of antimalarial agents such as quinine obtained from *Cinchona* species and artemisinin obtained from *Artemisia annua* (Qing hao) from medicinal plants also encouraged the possibility of finding new antimalarial drugs from plant sources (20).

Hence it is possible that the investigation of traditional medicinal plants used in the treatment of malaria may lead to the discovery of new antimalarial compounds . Traditional knowledge can be a fundamental starting point for chemotherapeutic research. A principal approach to chemotherapeutic research against malaria consists of investigating the traditional medicinal plant based antimalarials . The strategy of developing new drugs based on medicinal plants has an advantage over random screening, since it is guided by experience from a long history of clinical practice. Only 1% active plants among 300 randomly selected species which were tested, nearly 20% were found to be active among less than 50 plants based on traditional knowledge, identified up to the present (21). Plants investigated for pharmacologically active compounds are usually selected on the basis of ethnomedicinal information as there is a correlation between biological activity and the traditional use of the plant . In developing a drug from a plant attempts may be made to produce chemical analogues of the active principles with enhanced

antiplasmodial activity and reduced host toxicity drug research over the last two decades (22). The investigation of a range of plants from various countries used in traditional medicine for the treatment of malaria has led to the discovery of a large number of antimalarial compounds with significant structural variety. Examples of compounds with antimalarial activity isolated from traditional medicinal plants are quinines, triterpenes, sesquiterpenoids, quassinoids, liminoids, alkaloids and coumarins (23).

The urgency generated by drug resistant strains of malaria has accelerated antimalarial drug research over the last two decades . In malaria therapy, 11 drugs out of the antimalarials included in WHO therapeutic schemes for malaria treatment are natural products or their analogues or were design-based on the pharmacophores from natural products .The great significance of plant-derived drugs for the treatment of the disease is highlighted by quinine (derived from Cinchona tree), artemisinin (derived from Artemisia annua) and atovaquone , which is a synthetic compound analogue of lapachol from the Tabebuia species (24).

A number of in vivo and in vitro studies have been conducted to evaluate antimalarial activities of Ethiopian medicinal plants, Animut (25) showed that the water extracts of V. amygdalina leaf and methanol extract of Croton macrostachyus fruits have significant suppressive effect against P. berghei in vivo. Teklemariam (26) evaluated the antimalarial activities of *Moringa stenopetala, W. somnifera and V.amygdalina* against P.berghei in mice model showed valid results., Dikasso et a. (27) evaluated . The hydroalcoholic extracts from the roots and aerial parts of *Asparagus africanus* Showed significant antimalarial activity in the Swiss albino mice by 46.1`% and 40.7% respectively

1.2 Literature Review

1 .2.1 The family Rosacea

Rosaceae, in the order Rosals, is a large family containing more than 100 genera and 2,000 species of herbs, shrubs and trees of economic value, both for food (e.g., fruit trees including plums, apples, pears, loquats, blackberries and strawberries) and as ornamentals (e.g., flowers of the genus *Rosa*) . This family is represented on all continents except Antarctica, but the majority of species are found in Europe, Asia and North America (28).

1.2.2 *The Genus Hagenia*

Hagenia abyssinica (Bruce) J. F. Gmel is commonly known as Kosso in Ethiopia belongs to a monotypic genus in the family Rosaceae. It is a deciduous tree with distinct male and female trees, both of which are endowed with colourful flowers. The tree attains heights of up to 20 m, with short trunk and thick branches.The flowers are greenish, or white, turning reddish with maturity in female flowers. In Ethiopia, *H. abyssinica* was once abundant in the semi-humid mountain woodlands with the altitudinal range of between 2,450 and 3,250 m .The species occurs naturally within the undifferentiated Afromontane .The tree is so dominant in the mountains of tropical Africa. Outside Ethiopia, *H. abyssinica* is reported from the mountainous regions of Kenya, Tanzania, Malawi, Zimbabwe, Zambia, Zaire, Burundi, Uganda & Sudan (29).

Fig. 3 *Hagenia abyssinica (Bruce) J.F*

1.2.3 Medicinal Use of H. abyssinica

The female flowers of Kosso have long been used to expel tapeworm, a very common infestation among Ethiopians due to the age-long practice of eating raw beef by a large sector of the population (30,31).The traditional healer claims that kosso are also useful in treatment of high blood pressure , Fever/cough, Intestinal worms (tape worm) Stomachache, Diarrhea , healing of wound, Typhoid ,Cold (bronchitis) ,Epilepsy ,Livestock disease (thin/skinny body) , Evil eye ,Hepatitis, Sexually Transmitted Diseases (STDs), Throat disease, Proble ms related to Bile Cancer (mixed with other plants), Dermatology, Malaria ,and diabetes (32).

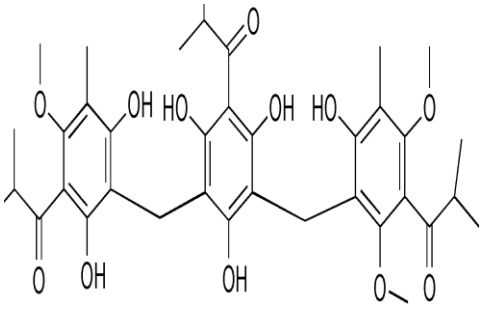
In East Africa the roots of *H.abyssinica* are cooked with meat and the soup drunk against general illness and malaria, besides its use as an anthelmintic. It is used in the treatment of syphilis together with powdered bark of *Albizzia anthelmintica* and also Against scrophulous tumour and cough. Between 1817 &1954, *Hagenia abyssinica* (Bruce) J. F. Gmel (kosso) was listed in the pharmacopoeias of 29 countries(30,31).

Women uses the bark *of H. abyssinica* during childbirth to prevent blood clotting and also uses to treat skin diseases .The seeds and the resin are toxic and are used as a fish poison. decoction of the roots is used as a laxative. oil of the seed is used against ear-inflammations.The soup of the leaves for headaches; inhaling smoke from burning leaves or drinking the leaf-sap is said to relieve pains of a woman in labour;chewing the root, bark or wood relieves toothache. A decoction of the leaves or the ash of burned leaves, is used against coughs. Besides being a source of medicine, *Hagenia abyssinica* has been utilized for various other purposes such as construction, furniture, fuelwood, and soil fertility management. As a result of its enormous significance,H. abyssinica is one of the endangered tree species in the country due to over exploitation (29,33).

*1.2.*4 The Chemistry of Hagenia abyssinica

Several secondary metabolites characterize the chemical composition of the *Hagenia abyssinica.* As active principles in this species, four phloroglucinol derivatives have been isolated.These are kosotoxin, protokosin,koisdine and kosine . The constituent responsible for its medicinal value is the phenolic compound, Kosin which is isolated from the ether extracts of the female flowers of *Kosso.*Kosins exhibits comparable potency as the marketed drugs, Dicholorophin &Nicolsamide.

Male flowers are not used traditionally as taeinicides due to their alleged high toxicity (34).The bitter principle called α kosin and β kosin, which are found in kosso, are thought to be decomposition products. Kosotoxin, a yellow amorphous powder possibly related to filicia acid and Rottlerin, is the principal constituent of kosso Where as protokosin and kosidin are inactive colorless bodies found in the extract of kosso . it also have Volatile oil, a bitter acrid resin, and tannic acid.(35) The methanol extract has been also isolated quercetin 3-*O*-β-glucuronide ,quercetin 3-*O*-β-glucoside , rutin and quercetin glycuronide (36).



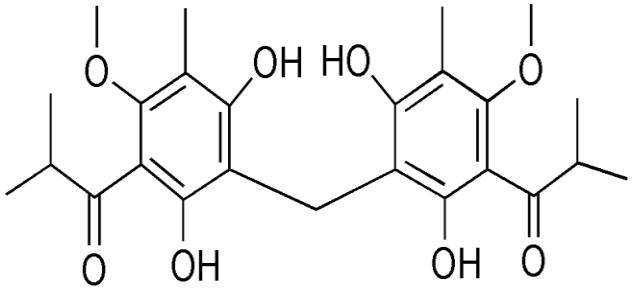


Figure .4 Protontoxin kosin

1.2.5 Pharmacologic Activity

Spasmolytic activity :- Kosso relieve non specific abdominal complaints which could arise from high secretion of acetylcholine and histamine in the gastro intestinal tract (37).

Kosin isolated from *Hagenia abyssinica*, showed anthelmintic as well as cytotoxic activity against a panel of three transplantable murine colonic adenocarcinomas with varying growth characteristics and morphology . Significant reductions in colony formation were observed tumour cell (38) .The hexane extracts of leaves of H. abyssinica were the most active extracts with the highest zones of inhibition against S. aureus, MRSa and P. aeruginosa (39).

1.3 Justification of The Study

Antimalarial drug resistance has emerged as one of the greatest challenges facing malaria control Today.Drug resistance has been implicated in the spread of malaria to new areas and played a significant role in the occurrence and severity of epidemics.The spread of multi drug-resistant *P. falciparum* has highlighted the urgent need to develop new antimalarial drugs ,that prevent transmission, stop relapse and provide long-duration molecules for chemoprotection against new infections and preferably inexpensive drugs that are affordable for developing countries, where malaria is prevalent (40,51).).

Globally, over 1000 plants have been used as potential antimalarials in resource-poor settings due to fragile health-care systems and lack of accessibility and affordability of artemisinin-based combination therapies (ACTs).Those plant have been in the past the source of some of the most successful antimalarial agents such as the quinolines and the endoperoxide artemisinin(42). One of the areas for the search for new anti malaria is the traditionally claimed antimalarial plants from the African flora. Studies have been conducted on traditionally claimed medicinal plants in Ethiopia and elsewhere for scientific validation. This is because they have been part of human life since time immemorial; & a number of plant products have been in extensive use in ethnomedicine (43).Finally Natural products have made and continue to make an immense contribution to malaria chemotherapy either directly as antimalarial agents or as important lead compounds for the discovery of more potent antimalarials. In the endemic area where malaria prevails, traditional herbal medicines are often used for antipyretic therapy and antimalarial therapy. However, very little scientific information is available to assess the efficacy of these herbal remedies. Therefore, it is important to investigate the safety and efficacy of antimalarial activities of medicinal plants that have a traditional claim in order to determine their potential as sources in the development of new antimalarial drugs. That is why this study is undertaken to evaluate the in vivo antimalarial activities of crude methanolic stem bark extracts of *Hagenia abyssinica* against *P.berghei* in laboratory bread Swiss albino mice to further substantiated the traditional use for malarial treatment (40,42).

2. OBJECTIVES

2.1 General objective

- To evaluate the anti malarial activity ,acute and sub acute toxicity of the crude haydroalcoholic extracts of the Stem bark of *Hagenia abyssinica* (Bruce) J. F. Gmel (Rosaceae) against *P.berghei* in swiss albino mice.

2.2. Specific objectives

- To assess the acute and sub acute toxicity of crude methanolic extracts of the Stem Barks of *Hagenia abyssinica* (Bruce) J. F. Gmel (Rosaceae) in Swiss albino mice.
- To identify secondary metabolites in crude methanolic extracts of the stem bark of *Hagenia abyssinica* (Bruce) J. F. Gmel (Rosaceae).
- To evaluate the antimalarial effects of crude methanolic extracts of the Stem Bark *of Hagenia abyssinica in* Swiss albino mice infected with *P. berghei*.

3. Materials and Methods

3.1 Materials and Animals

3.1.1 Plant Material

The stem barks of *H. abyssinica* was collected in December 2013 from kossoye kebele 780 Km North west of Addis Ababa, Central Ethiopia. The authenticity of the plant material were confirmed,by the National Herbarium, Department of Biology, Addis Ababa University, where voucher specimen (collection number Tm 01) deposited.

3.1.2 Reagents and Drugs

Table.1 chemicals and reagents used in the experiments.

Chemicals	Manufacturer	Obtained from	Country
Chloroquine	EPHARM	Gondar Private pharmacy	Ethiopia
Methanol	Avishkar Labtechche	PFSA, Addis Ababa-Ethiopia	India
Ammonia	BDH Chem ltd Poole	Dep`t of pharmacognocy, UOG	England
Ferric chloride	AvishkarLabtechche	Dep`t of pharmacognocy, UOG	India
Glacial acetic acid	CDH Labor reagent	Dep`t of pharmacognocy, UOG	india
Sulfuric acid	Supertek chemicals	Dep`t of pharmacognocy, UOG	India
Acid alcohol	AvishkarLabtechche	Dep`t of pharmacognocy, UOG	India
Meyers reagent	Supertek chemicals	Dep`t of pharmacognocy, UOG	India
Draggendorffs reagent	Supertek chemicals	Dep`t of pharmacognocy, UOG	India
ninhydrin reagent	Supertek chemicals	Dep`t of pharmacognocy, UOG	India
Lead acetate	Supertek chemicals	Dep`t of pharmacognocy, UOG	India
Hydrochloric acid	Supertek chemicals	Dep`t of pharmacognocy, UOG	India
Nitric acid	Supertek chemicals	Dep`t of pharmacognocy, UOG	India
Giemsa	AvishkarLabtechche	Dep`t of pharmacology, UOG	India
Sodium citrate	AvishkarLabtechche	Dep`t of pharmacology, UOG	India

All the chemicals were analytical grade & most of them were obtained from school of pharmacy in UOG.

3.1.3 Experimental Animals

Male and female Swiss albino mice weighing 24-30 g and age 6-8 weeks were obtained from university of Gondar , animal house, Gondar . All animals were housed in an air-conditioned room and were allowed to acclimatize for one week before the study. The animals were kept at room temperature and were exposed to a 12 hour light/dark cycle. All the experiments were conducted in accordance with the internationally accepted laboratory animal use, care and guideline(44). Before and during the experiment, the mice were allowed free access to standard pellets and water *ad libitum*.

3.1.4Parasite

Plasmodium berghei ANKA strain (chloroquine sensitive), was purchased from Aklilu Lemma Institute of Pathobiology. The parasite was subsequently maintained in the laboratory by serial blood passage from mouse to mouse on weekly basis.

3.2. Method

3.2 .1 Preparation of Plant Material

The bark of *H. abyssinica* was cleaned, air-dried at room temperature and crushed into coarse powder. The powdered plant material (100 g) was macerated in one liter 80% methanol (Lab tech chemicals, CASR NO 67-56-1 A.B.NO- 130507) for 72 h with occasional stirring. The filtrate was separated from the mark using Whatman filter paper 18cm diameter and 0.1μm pore diameter and the mark was re-macerated two times. The filtrates were combined and dried in an oven (Gallenkamp, England) at the temperature not exceeding 40℃. The dried extract was transferred into vials and were kept in a desiccators until use.

3.2 .2 Preliminary Phytochemical Screening

The 80% methanol extract of *H.abyssinica* was screened for the presence of alkaloids, flavonoids, polyphenols, tannins, saponins, terpinoide, antraquinone, resin and steroidal compounds(49,50).

3.2.3 Acute oral Toxicity Test

A preliminary toxicity study was designed to demonstrate the appropriate safe dose range that could be used for subsequent experiments rather than to provide complete toxicity data on the extract and the acute toxicity of *Hagenia abyssinica*. The methanolic stem bark extract was carried out using OECD-425 guide lines .The study mice was fasted for 4 hours.A single limit dose of 2g/kg stem bark extract was administered to a total of 5 female Albino mice(22-25g each) within 24 hour interval. The mice were observed for 14 days to asses signs of toxicity and death (45).

3.2.4 Sub Acute Toxicity Test

The crude methanol extracts of *Hagenia abyssinica* intended for the antimalarial test against P. berghei were evaluated for its toxicity in non-infected male and female Swiss albino mice aged 6-8 weeks and weighing 27-32 gram. For the test, 12 mice were used by randomly dividing them into four groups of 3 mice per cage. Before oral administration of a single dose of each extract, the mice were fasted for 3 hours . For subacute toxicity studies weight and PCV were measured before and after treatment. The extracts in each case was administered orally in an increasing dose related manner for four days (i.e. D0 to D4) using gavage. Then, the mice in group one, two and three were given orally 100, 200 and 400mg/kg body weight in single dose volume of 0.2 ml of each extract, respectively. The mice in the control groups received 0.2 ml of respective vehicle of each extract. Then, the mice were observed continuously for one hour after the treatment; intermittently for 4 hours, and there after over a period of 24 hours.The mice were also observed for gross behavioral changes such as feeding, lacrimation mortality and other signs of toxicity manifestations for 24 hours.

In subacute toxicity studies, the mice were closely observed for a week and data were recorded on day 0 and day 4 (after 12 hours of the last dose given). The subacute toxicity of each extracts was evaluated in terms of gross behavioral changes, mortality, weight loss, Organ weight loss and reduction in PCV. The body weight of each mouse was determined in grams using weighing balance. The packed cell volume was determined by microhaematocrit reader. The mice were dissected and liver, kidney lung and heart were excised and weighed.

Data on weight, Organ weight, packed cell volume, were obtained on day zero (D0) and day four (D4). For all experimental mice, the averages of all parameters before treatment (D0) and after treatment (D4) were compared with control group and among different dose levels (15).

3.2.5 Antimalarial Activity Test of the Crude Extract

The standard four-day suppressive method was used (46,47). Blood was taken from a donor mouse by cardiac puncture with approximately 30% parasitemia and diluted in physiological saline to 5x107 parasitized erythrocytes per ml. Swiss albino mice weighing 26-32g were infected with 0.2 ml (1x107 parasitized erythrocytes) *P. berghei* intraperitoneally (i.p) & randomly divided into five groups of five mice per cage with three test groups and two control groups (each for chloroquine as a standard drug and distilled water as a negative control). The test extract was prepared in three different doses (100 mg/kg, 200 mg/kg, and 400 mg/kg of body weight) and chloroquine phosphate USP (EPHARM B. No-1041213), at 10 mg/kg in a volume of 0.2 ml. Each extract or the standard were administered as a single dose per day. The extract and the drug were given through oral route by using standard oral gavages. Treatment was started 3 hour after infection on day 0 and were then continued daily for four days (i.e. from day 0 to day 3). On the fifth day (D4) thin and thick smears of blood films were obtained from the peripheral blood on the tail from each mouse. The smears was placed on microscopic slides (Westtmed Praxis, Germany), was fixed with methanol (EPHARM B. No-7051213) and stained with 10% Giemsa at pH 7.2 for 15 minute.

The parasitaemia level was determined by counting the number of parasitized erythrocytes out of six random fields of the microscope (Olympus 6V20 WHA2, Japan). Average percent parasitaemia and suppression was calculated by using the following formula (48).

$$\text{Parasitaemia} = \frac{\text{Number of parasitized RBC}}{\text{Total number of RBC count}} \times 100$$

$$\% \text{ Suppression} = \frac{\text{Mean parasitemia of negative control - Mean parasitaemia of treated}}{\text{Mean parasitemia of negative control}} \times 100$$

3.2.6. Determination of Body Weight and Temperature

The body weight of each mouse in all group was taken before infection (day 0) and on day 4. The rectal temperature of the mice was measured with a digital thermometer before infection, three hours after infection and then daily up to day 4 to see the effect of the extracts on body temperature.

3.2.7 Determination of Mean Survival Time

Mortality was monitored daily and the number of days from the time of inoculation of the parasite up to death was recorded for each mouse in the treatment and control groups throughout the follow up period. The mean survival time (MST) for each group was calculated as:-

$$MST = \frac{\text{sum of survival time of all mice in a group(daily}}{\text{Total number of mice in the group}}$$

3.2.8 Data Quality Control

Data quality was controlled by use of appropriate number of animals, randomization , coding and use of instruments and chemicals from appropriate sources.

3.2.9 Data Analysis

Data was analyzed using Windows SPSS Version 16. The one-way analysis of variance (ANOVA) followed by Tukey‟s HSD *post-hoc* test, was used to compare results among and within groups . Paired *t-test* were also used to compare some parameter between initial and final results. The results were considered significant when $P<0.05$.

3.3 Ethical Considerations

The animals were handled according to the international animal care and welfare guidelines (35). The ethical clearance were requested and obtained from research and ethics review committee of University of Gondar.

4. Result

4.1 Percentage Yields of Crude Extract

The 80% methanol bark extract of *Hagenia abyssinica* gave a redish brown solid poweder. The percentage yields of the crude extract was 4.68 %.

4.2 Phytochemical Study

The Phytochemical tests revealed the presence or absence of major secondary metabolites in the extracts of the stem barks of *H. abyssinica* by using specific chemicals and methods. The results obtained from the tests were summarized .

Table 2: Secondary metabolites of methanol extract of the stem bark of *H.abyssinica.*

Secondary metabolite	Reagent	Result
Alkaloids	Mayer's Test /Wagner's Test:	+Ve
Antraquinone	Ninhydrin Test:	+Ve
steroides	Molisch's Test /Benedict's Test	-Ve
Cardiac glycosides	Legal's Test	-Ve
Terpinoide	Copper acetate Test	+Ve
Flavonoids	Alkaline Reagent Test /Lead acetate Test	+Ve
Phenols	Ferric Chloride Test	+Ve
Phytosterol	Salkowski's Test /Salkowski's Test	+Ve
Resin	Resin test	+Ve
Saponines	Froth Test /Foam Test	-Ve
Tannins	Ferric Chloride Test	+Ve

Note: - -ve negetive +ve positive

4.3 Toxicity Test

The acute toxicity study indicated that of the extract did not cause mortality of mice within 24 hrs up to 2000mg/kg. Gross physical and behavioral observation of the experimental mice also revealed no visible signs of acute toxicity like lacrimation, hair erection, tremors, convulsions, salivation and reduction in their motor and feeding activities. They were physically active.

4.4 Sub-Acute Toxicity

Table 3. Sub-acute toxicity tests of the hydroalcoholic stem bark extract of Hagenia abyssinica on body weight and PCV.

Dose(mg/kg/day)	Parameters	D0 +SEM	D4 +SEM	% change	p-value
100	Weight (g)	29.3±4.3	25.45±4.1	- 13.14%)	0 .025
	PCV (%)	57.67± 3.05	64±3.51	+10.97%	0 .089
200	Weight (g)	30.4±2.5	25.4±.49	-16.3%	.007
	PCV (%)	59.66±2.2	63.±3.0	5.58	0 .063
400	Weight (g)	32.33±2.73	27.6±3.59	- 17.14	0 .037
	PCV (%)	66.33±3.38	71.33±.66	+7.54	0.342
Vehicle	Weight (g)	27.67±3.61	25.3±3.56	- 8.56	0 .149
	PCV (%)	60.00±.577	59.6±3.5	0 .5	0.874

SEM – standard error of mean P<0.05 was considered significant

Day 0 = day infection was initiated Day 4 = 5th day of infection

As shown in Table- 3 statistically significant differences (P<0.05) was observed in weight when we compare in each group between pre-treatment (D-0) and post-treatment (D-4) but not significant weight variation observed in control group(P>.05). Similarly, slight increase in PCV was recorded in extract treated group while there was very small decrease of this parameter on day 4 relative to day 0 in negative control group, which was statistically not significant(p>.05). The sub-acute toxicity test revealed the non toxic effect of the extract at the given dose.

Table 4:- Sub-acute toxicity tests of the hydroalcoholic stem bark extract of Hagenia abyssinica on organ weight in Swiss Albino Mice

Organs	Control	100mg	200mg	400mg
Liver	1.31±0.18	1.58±0.120	1.18±0.22	1.49±0.07
Kidneys	0.32±0.06	0.39±0.05	0.21±0.06	0.33±0.05
heart	0.14±0.01	0.16±0.01	0.13±0.03	0.15±0.01
lung	0.27±0.01	0.34±0.04	0.21±0.04	0.25±0.01

As shown in table -4 the organ weights of liver, kidney, heart and lung did not record any significant alterations ($P>0.05$) in all the treated groups when compared to control group.

Generally,Sign of toxicity such as change in animal behaviour, lacrimation, weight loss, hair erection and mortality were not recorded both in acute and sub-acute toxicity testing. This fulfils the criteria set by OECD -425, lack of acute toxicity by the plant extract.

4.5 Effect of Extract on Parasitimia

The result of Antimalarial suppressive test of crude methanol extracts of H. abyssinica against drug sensitive , *P.berghei* (ANKA strain), in mouse possesses blood schizontocidal activity in the early infection as shown in table -5 .The multiple comparison tests indicated that all the mice treated with the three extracts resulted in reduced parasite load as compared to their respective negative control groups. The extracts did not clear the parasite completely, where as positive control groups treated with CQ phosphate, used as a standard antimalarial drug, at daily dose of 10mg/kg body weight totally cleared the parasite on day four under identical condition. Hence, the extract was less potent than CQ phosphate.

Table:- 5 Antimalalarial activities of hydroalcoholic stem bark extract of H.abyssinica in Swiss albino mice infected with P. berghei

Drug/extract	**N**	Average % Parasitaemia	Average% suppression
Control	5	24±1.87	0.0
100mg/kg	5	8.33±1.67	65.29±1.7
200mg/kg	5	5±1.45	79.6±2.2
400mg/kg	5	4±1.87	83.33±4.3
Chloroquine	5	0.0	100±0

N – number of mice in each group $P<0.01$ was considered significant

Hydroalcoholic stem bark extract of H. abyssinica showed statistically significant (P<0.001) percentage of Parasitaemia against *P.berghei* at all dose levels (100-400mg/kg) tested mice compared to the mice in the negative control group on day 4. The average percent of parasitaemia observed at a highest dose (400 mg/kg/day) was found to be 4.0±1.87 with 83.33% of chemosuppression. A dose of 200 mg/kg/day of the extract showed 5.0±1.87% parasitemia with 79.6% chemosuppression, while the lowest dose (100 mg/kg/day) lowered the average percent parasitemia to 8.33±1.67 with chemosuppression of 65.29% where as the corresponding figure in the negative control group was 24.0±01.87%. This reveals that the parasitemia in the experimental mice were lower than that of the negative control groups and the extract has dose dependent activity.

Comparasion of percentage of Parasitemia of the lowest dose (100 mg/kg/day) with distil water and CQ phosphate it is statically significant with p-value of P<0.001 and P<.022 respectively but when we compare it with 200mg and 400mg it is not statically significant with p- value of p> .651 and p> .411 respectively. When we compare 200mg with 400mg and CQ phosphate it is not statically significant with p- value of p> .989 and p> .166 respectively but statically significant with distil water with p-value of P<.001. When we compare 400mg with CQ phosphate it is not statically significant with p- value of p> .351 but statically significant with distil water P<.001. .

4.6 The Effect of H.abyssinica Extract on Body Temperature

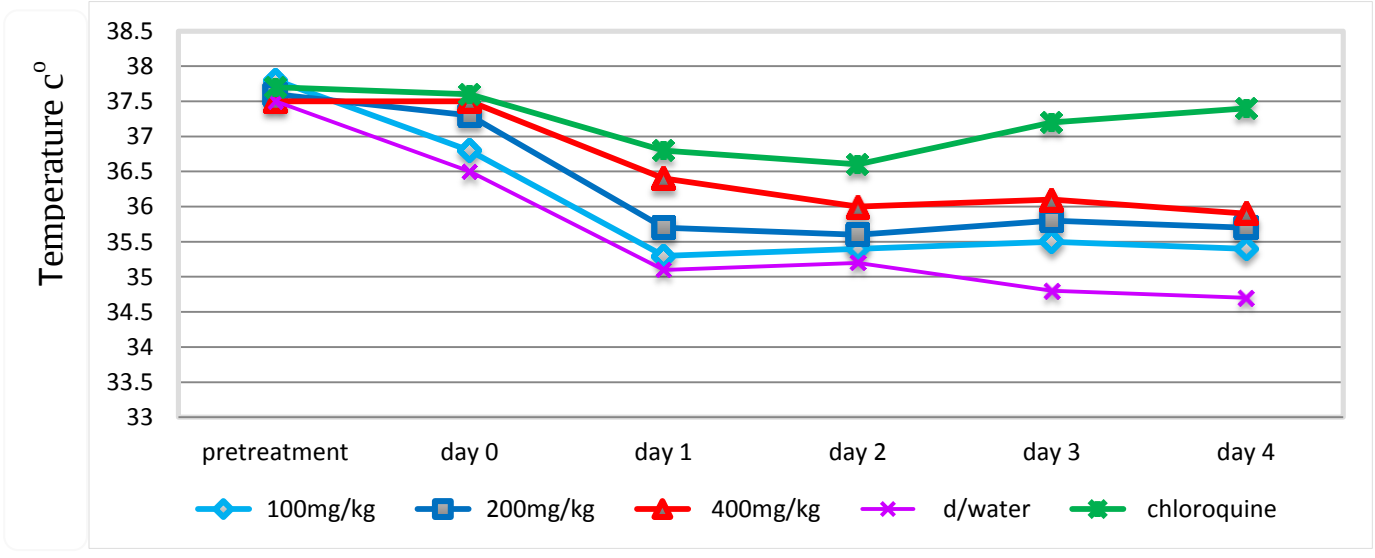


Figure.5 Effect of extract on body temperature

In all three models, the hydromethanolic stem bark extract of *H.abyssinica* did cause prevention of rectal temperature reduction of *P. berghei* infected mice when we compare with distle water treated mice but not statically significant (p>.05). The standard drug, chloroquine 10 mg/kg had showed statically significant(p=.036) activity in prevention against rectal temperature reduction when compared to the negative control as shown in the figure - 5 .

4.7 Effect of the H.abyssinica Extract on Body Weight

Table 5. The effect of the extract on body weight

Drug/extract	Dose(mg/kg/day)	N	Weight D0±SEM	Weight D4±SEM	% change	p-value
H.abyssinica	100	5	30.3±.73	25.5±.63	–15.8%	.001
H.abyssinica	200	5	27.4±.63	23.3±1.14	–14.96	.033
H.abyssinica	400	5	29.9±.66	25.0±.38	–16.38	.003
Chloroquine	10	5	24.6±.66	22.4±.92	–8.9	.223
Vehicle	0.2 ml	5	24.4 ±1.30	22.4 ±.92	–8.2	.140

Day 0 = day infection was initiated , Day 4 = 5th day of infection – = Decrease in average body weight
N – number of mice in each group P<0.05 was considered significant

In 4-day suppressive test all dose levels of extract of *H.abyssinica* had shown body weight loss between days 0 and 4 in both control and extract treated groups. All mice had lost some of their body weight but still the control group mice had better body weight status than the extract treated mice. There was a significant (p<0.05) loss of body weight between days 0 and 4 in extract treated groups but not statically significant(p>.05) in positive and negative control groups as shown in table 5. All dose of the crude extract was found to significantly reduce body weight of healthy mice.

4.8 Effect of the H.abyssinica Extract on Survival Time

Table 6**.** Mean survival time of *Plasmodium berghei* infected mice after treatment with the hydroalcoholic bark extract of *Hagenia abiyssinica*

Drug/extract	N	Dose (concentration) mg/kg/day	Survival time ± SEM	p-value
Vehicle	5	0.2 ml	6.8±0.37	-
H.abyssinica	5	100	10.0 ±.58	.015
H.abyssinica	5	200	12.2±1.17	.000
H.abyssinica	5	400	15.2±.97	.000
chloroquine	5	10	28.0±.00	.000

SEM – standard error of mean N – number of mice in each group P<0.05 was considered significant

The mean survival time of mice of the given extract was shown to be 10.0±0.57, 12.40±1.16, 15.2±0.97 days, for doses of 100, 200 and 400 mg/kg/day, respectively, which was statistically significant (P<0.05) when compared to Vehicle treated mice which was 6.8±0.37 days as shown in table 6. The mean survival time increases as dose the increases which is dose dependent. All animals treated with the standard drug, chloroquine 10 mg/kg/day, survived more than 28 days.

5. Discussion

Most common antimalarial plants used to treat malaria in traditional medicine contain secondary metabolites .The phytochemical screening of hydromethanolic stem bark extract of *H.abyssinica* indicated the presence of different secondary metabolites that have antiplasmodial activity in other plants like flavonoids(51), terpenoids(52), phenols(53), phytosteriods,Tannins , alkaloids(54),Anthraquinones(55).which have been suggested to be responsible for antiplasmodial activity of stem bark of H.abyssinica. As the antiplasmodial activity observed in many plants was considered to result from single or combined action of these metabolites, same could be said for the present study.

The phenols present in this plant which have antioxidant effect (56) may also contribute to the antimalarial activity due to inhibition of haem polymerization. The phytosteroids and flavonoids detected in this plant could as well be responsible for the antimalarial effect as these metabolites have been proved to possess potential immunomodulatory effects in other plants (42).

The acute toxicity of *H.abyssinica* has been investigated to determine any adverse effect that may arise as a result of a short time mice exposure to the methanol extract H.abyssinica .None of the test mouse died or showed signs of acute toxicity within 24 hours of treatment with the test extracts. So the extracts were nontoxic to the test mice, as they did not show signs of acute toxicity within 24 hours at the doses of 2g/kg body weight .

Similarly, in sub acute toxicity study of the extracts revealed no signs of toxicity manifestations within 14 days at the dose levels tested. But causes significantly loss of weight at all dose levels compared to the controls. It may possibly indicate the appetite suppressive effects of the extracts, which would reasonably increase with increasing dose. This in turn can affect the feeding capability of the mice and could cause a relative reduction in body weight. which could be indicative of toxicity. However, the toxicity tests of the present study do not indicate any other toxicity manifestation at reasonably higher doses and no death was observed before five days. This shows that, all the test extracts were non-toxic to the test mice (27).

In this study, the anti-plasmodial activity of *H.abyssinica* was investigated using *in vivo* animal (mice) model. Mice were used to predict the antimalarial activities of the extract because Synthetic antimalarial drugs like chloroquine, mefloquine and more recently artemisinin have been investigated using mice model (46). The In-vivo Plasmodium models such as P.berghei,

P. vinckei and P. yoelii are firmly established models in anti-malarial drug discovery . The parasite *P. berghei* was used to predict treatment outcomes since it is accessible and sensitive to chloroquine , it was used as reference drugs(57). When a standard antimalarial drug is used in mice infected with *P. berghei*, it suppresses parasitemia to non-detectable levels , which is in agreement with the effects of chloroquine in this study (58).

The 4-day suppressive test is a standard test commonly used for antimalarial screening, and the determination of percent inhibition of parasitemia is the most reliable parameter. It also provides a preclinical indication of potential bioactivity of the test sample (59).The life prolonging potential of the methanol extract of *H.abyssinica* in malaria infected mice could be seen from its prolongation of the mean survival time. The mice treated with the extract had significantly longer survival time than the negative control mice, which did not survive longer than 6.8 days. This effect has value in patient care whereby the extracts could be used until such time that curative medication can be found for proper treatment of malaria patients. According to Peters (47) The mean survival time of mice is considered as an evidence for the antiplasmodial activity of the extract. There was lower parasitaemia level in the extract administered group and longer survival time of the mice at the highest doses of the extract indicating strongly associated with a dose dependent pharmacological activity and this was in agreement with other *in vivo* antimalarial test (48).

In 4-day suppressive test, it was only chloroquine treated mice that prevented body weight loss ($P>0.05$) but all the extract treated mice had shown a statically significant ($P<0.05$) weight loss when compared to negative control. Body weight loss in extract treated mice might be possibly due to depressing effect of the crude extract on feed intake or appetite and this result is in agreement with that of a previous study on other medicinal plant (41). The result of the present study on body weight, however, is not in agreement with that of of Dicasso. (26). The stem bark Extract of *H.abyssinica* showed anti-malarial activity against *P. berghei* infection in mice as evidenced by the percentage of parasite inhibition. As shown from the results of the *in vivo* antiplasmodial studies, the methanol extract of *H.abyssinica* exhibited highest suppressive activity on *P. berghei*. The plant extracts are less effective when compared to that of the standard antimalarial drug, chloroquine phosphate. The effects on parasitemia in this study are similar to the ones reported by previous studies such as on *Solanum surattense (60), Piliostigma thonningii* schum *(61) Maerua crassifolia forssk* (62), *Croton macrostachyus Hocsht* (57*)* and

However, relatively lower antiplasmodial activities than the present result have been reported on *Khaya grandifoliola Welw* (63), *Dodonaea angustifolia* (41).

From literature an *in vivo* anti-plasmodial activity can be classified as moderate, good and very good if an extract displayed percent parasitemia suppression equal to or greater than 50% at a dose of 500 mg, 250 mg and 100 mg/kg body weight per day, respectively. Based on this classification, the parasitemia chemosuppression was greater than 50% at the lowest doses (100 mg/kg body weight). It can thus be generally concluded that the hydromethanolic stem bark extract of *H.abyssinica* exhibited a very good anti-plasmodial activity(57).

6. Conclusion

From this study, it can be concluded that the Acute and subacute toxicity studies showed the non toxic effect of the methanol extracts of Stem bark of H. abyssinica. Ethnobotanical knowledge was sufficiently reliable for identifying plant extracts with antiplasmodial activity. The results obtained from the present work support the traditional use of H. abyssinica against malaria and confirmed the Methanol extract of H. abyssinica has parasite suppressive effects against Plasmodium berghei infected Swiss albino mice in a dose related manner .Antimalarial activities as well as the lack of toxicity of the extracts suggest its ethnopharmacological usefulness as anti malarial. Because of the antimalarial activity of the methanol extract, the plant serve as a potential source for isolation of lead antimalarial compounds.

7. Recommendation

- The identification & validation of antimalarial active compounds of these traditional plants should involve researchers from different disciplines in order to achieve better results
- The active components of the plant extracts need to be purified by bioassay guided isolation method and further work must be undertaken to isolate the active compounds responsible for the observed antimalarial activity of the extracts.
- pharmacological and toxicological studies should be done before drug development.
- Further investigation as a potential antimalarial in the Atotus Monkey (*Aotus trivirgatus*) against *P. falciparum* infection will be justifiable.
- In vivo antimalarial testing should be done on other parts of the plant like flower,leaf,root by using different solvents.

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DECLARATION

I, the under signed, declare that this is my original work and has never been presented in this or any other university and that all the source materials which are used for the thesis have been properly acknowledged.

Name :- Tafere Mulaw

Signature ----------

Place: Gondar, Ethiopia

Date of Submission June , 2014

9. Appendices

Annex 1: List of Equipments:-

- ✓ Electrical balance, disposable glove, oral gavages, measuring cylinder, syringe with needle, beakers, mortal and pestle, Buckner funnel, what man no.1 filter paper, surgical blade ,slides, pipette, scissor and microscope, digital thermometer

Annex 2:Phytochemical screening procedures:-

1. Detection of alkaloids:Extracts were dissolved individually in dilute Hydrochloric acid and filtered.

a. Mayer's Test**:** Filtrates were treated with Mayer's reagent (Potassium Mercuric Iodide).Formation of a yellow coloured precipitate indicates the presence of alkaloids.

b. Dragendroff's Test:Filtrates were treated with Dragendroff's reagent (solution of Potassium Bismuth Iodide). Formation of red precipitate indicates the presence of alkaloids.

2. Detection of glycosides:Extracts were hydrolysed with dil. HCl, and then subjected to test for glycosides.

a. Modified Borntrager's Test:Extracts were treated with Ferric Chloride solution and immersed in boiling water for about 5 minutes. The mixture was cooled and extracted with equal volumes of benzene. The benzene layer was separated and treated with ammonia solution. Formation of rose-pink colour in the ammonical layer indicates the presence of anthranol glycosides.

3. Legal's Test: Extracts were treated with sodium nitropruside in pyridine and sodium hydroxide. Formation of pink to blood red colour indicates the presence of cardiac glycosides.

4. Detection of saponins:-Foam Test: 0.5 gm of extract was shaken with 2 ml of water. If foam produced persists for ten minutes it indicates the presence of saponins.

5. Detection of phytosterols :- Salkowski's Test:Extracts were treated with chloroform and filtered. The filtrates were treated with few drops of Conc. Sulphuric acid, shaken and allowed to stand. Appearance of golden yellow colour indicates the presence of triterpenes.

6. Detection of phenols :- Ferric Chloride Test:Extracts were treated with 3-4 drops of ferric chloride solution. Formation of bluish black colour indicates the presence of phenols.

7. Detection of tannins :-Gelatin Test: To the extract, 1% gelatin solution containing sodium chloride was added. Formation of white precipitate indicates the presence of tannins.

8. Detection of flavonoids

a) Alkaline Reagent Test:Extracts were treated with few drops of sodium hydroxide solution. Formation of intense yellow colour, which becomes colourless on addition of dilute acid, indicates the presence of flavonoids.
b) Lead acetate Test:Extracts were treated with few drops of lead acetate solution. Formation of yellow colour precipitate indicates the presence of flavonoids.

9. Detection of diterpenes:- Copper acetate Test: Extracts were dissolved in water and treated with 3-4 drops of copper acetate solution. Formation of emerald green colour indicates the presence of diterpenes

10. Detection of antraquinone:-:-1gram of extract is boiled for two minutewith five mlof 0.5N KOH and 0.5mlof 5% H_2O_2 mixture.after cooling ,the suspention is filtered through the glass wool. The filtrate is treated with six drops of acetic acid and the resulting solution is mixedwith five ml of tolune.The upper layer is separetedwith a pippat and transferred to a test tube and two ml of 0.5N KOH is added.ifared color appears in the aqueous layer ,anthranoids present.